

# Methylation and Expression Analyses of the 7q Autism Susceptibility Locus Genes *MEST*, *COPG2*, and *TSGA14* in Human and Anthropoid Primate Cortices

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## Key Words

Autism spectrum disorder • Brain evolution • Communication abilities • *COPG2* • Gene expression • Imprinting • *MEST* • Primate evolution • Promoter methylation • Social brain

## Abstract

The autism susceptibility locus on human chromosome 7q32 contains the maternally imprinted *MEST* and the non-imprinted *COPG2* and *TSGA14* genes. Autism is a disorder of the 'social brain' that has been proposed to be due to an overbalance of paternally expressed genes. To study regulation of the 7q32 locus during anthropoid primate evolution, we analyzed the methylation and expression patterns of *MEST*, *COPG2*, and *TSGA14* in human, chimpanzee, Old World monkey (baboon and rhesus macaque), and New World monkey (marmoset) cortices. In all human and anthropoid primate cortices, the *MEST* promoter was hemimethylated, as expected for a differentially methylated imprinting control region, whereas the *COPG2* and *TSGA14* promoters were completely demethylated, typical for transcriptionally active non-imprinted genes. The *MEST* gene also showed comparable mRNA expression levels in all analyzed species. In con-

trast, *COPG2* expression was downregulated in the human cortex compared to chimpanzee, Old and New World monkeys. *TSGA14* either showed no differential regulation in the human brain compared to chimpanzee and marmoset or a slight upregulation compared to baboon. The human-specific downregulation supports a role for *COPG2* in the development of a 'social brain'. Promoter methylation patterns appear to be more stable during evolution than gene expression patterns, suggesting that other mechanisms may be more important for inter-primate differences in gene expression.

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Genomic imprinting is the epigenetic marking of a subset of genes that results in the preferential or exclusive expression of 1 of the 2 parental alleles. Most imprinted genes are clustered with differentially methylated imprinting control regions. Parent-specific methylation patterns are established in the male or female germline, respectively, and then maintained after fertilization and during further development [Ferguson-Smith and Surani, 2001; Kelsey, 2007]. Many imprinted chromosomal domains contain both maternally and paternally ex-

pressed genes, often interspersed with non-imprinted genes. Imprinted genes are essential for the regulation of fetal and placental growth and somatic differentiation [Miozzo and Simoni, 2002; Reik et al., 2003]. To date, about 80 imprinted genes are known in human and other mammalian species (<http://igc.otago.ac.nz>; <http://www.geneimprint.org>). It is estimated that at least 100–200 imprinted genes exist in the mammalian genome [Murphy and Jirtle, 2003]. High-resolution expression analyses identified more than 1,000 loci with possibly preferential expression of the paternal or maternal allele in embryonic and/or adult mouse (*Mus musculus*) brain regions, implying a major role for genomic imprinting in brain development, neurological and behavioral functions [Gregg et al., 2010].

Genomic imprinting evolved in a common ancestor of marsupials and eutherians after divergence from the egg-laying monotremes, most likely because of a parental antagonism over the extraction of maternal resources for the offspring [Moore and Haig, 1991]. This hypothesis is mainly based on the finding that *IGF2* and the *IGF2* receptor are oppositely imprinted in therian mammals but not in monotremes and birds [Killian et al., 2000]. Many paternally expressed genes, such as *IGF2*, enhance fetal and placental growth by extracting maximal maternal resources, whereas maternally expressed genes such as *IGF2R* save maternal resources and restrict growth. This intraindividual conflict of the 2 parental alleles at imprinted loci may extend beyond the maternal-fetal relationship during intrauterine development. Imprinted genes that are expressed in the juvenile and adult brain have been proposed to be key players for the evolution of a ‘social brain’ [Ubeda and Gardner, 2010, 2011]. In utero, the fetus depends almost entirely on maternal resources, and the fetal-maternal interactions are biochemical in nature. However, after birth and in particular in the post-weaning period, both parents may contribute resources to the offspring, and parental care is mainly modulated by social interactions. In this context, it is interesting to note that approximately 10% of all mammalian but 40% of primate species show biparental care [Ubeda, 2008]. It is plausible to assume that imprinted genes have effects on adult cognition and behavior even after cessation of parental care. It has been proposed that psychosis and autism are disorders of the ‘social brain’ [Curley, 2011; Wilkins and Ubeda, 2011].

Communication abilities are of vital interest for the offspring to demand resources from its parents in the weaning and post-weaning periods. In human and non-human primates, gestures and vocalizations are key com-

ponents of social interaction. Intentional rather than reflective communication requires a theory of mind which enables an individual to assess the mental state of others [Tomasello, 2008]. This ability is severely impaired in patients with autism spectrum disorder (ASD) who display repetitive and stereotyped behavior, language impairment, and limited social interaction [Stefanatos and Baron, 2011]. Genome-wide association studies suggested an autism susceptibility locus on human chromosome 7q [IMGSAC, 1998]. The candidate region contains the maternally imprinted *MEST/PEG1* gene (mesoderm-specific transcript/paternally expressed gene 1). *Mest*-deficient female mice showed reduced maternal care for their offspring, providing a link between impaired imprinting and abnormal adult behavior [Lefebvre et al., 1998]. However, screening of candidate genes in the 7q autism susceptibility locus, including *MEST* and *COPG2*, did not identify any DNA sequence or imprinting mutations in autism families so far [Bonora et al., 2002]. Parent-of-origin linkage modeling at the 7q locus suggested the involvement of epigenetic mechanisms in the etiopathogenesis of ASD [Lamb et al., 2005; Schanen, 2006].

The appearance of advanced communication skills and in particular language abilities have been proposed as a driving force in the evolution of the human brain [Jerison, 1976]. Because the genetic differences between humans and chimpanzees are rather small [Varki and Altheide, 2005], the striking species differences, i.e. in communication abilities, are more likely to be due to changes in gene regulation rather than to structural changes in the gene products. In this study, we have analyzed the epigenetic regulation of 3 candidate genes in the 7q autism susceptibility locus, *MEST*, *COPG2*, and *TSGA14*, in human and anthropoid primate cortices. *MEST* is maternally imprinted in human and mouse and expressed particularly in mesodermal tissues [Nishita et al., 1996]. The coatmer protein complex, subunit gamma 2 (*COPG2*) gene overlaps the *MEST* 3'-UTR in a tail-to-tail fashion. It is expressed ubiquitously with a partial paternal imprinting pattern in mouse fetal tissues [Lee et al., 2000] and a partial maternal imprinting in human fetal tissues [Blagitko et al., 1999]. Other studies found bi-allelic expression in all human fetal and adult tissues, suggesting that human *COPG2* escapes genomic imprinting [Yamasaki et al., 2000]. The non-imprinted testis-specific gene A 14 (*TSGA14*) is located in head-to-head orientation upstream of *MEST* [Yamada et al., 2002]. It is highly expressed in human fetal testis and brain and encodes a centrosomal protein.

**Table 1.** Individuals examined

Species		Sex	Age, years	Age class	Cause of death
Human	HSA1	male	39	late adult	cerebral hemorrhage
	HSA2	female	24	early adult	sudden cardiac death
	HSA3	male	45	early mature	accident
	HSA4	male	40	late adult	suicide
	HSA5	male	40	late adult	traffic accident
	HSA6	male	54	late mature	accident at work
	HSA7	male	31	late adult	suicide
	HSA8	male	39	late adult	drug overdose
	HSA9	male	44	early mature	suicide
	HSA10	male	75	senile	intra-abdominal hemorrhage
	HSA11	female	83	senile	exhaustion
	HSA12	male	65	senile	cancer
	HSA13	male	41	early mature	heart attack
	HSA14	male	59	late mature	heart attack
Chimpanzee	PTR1	male	14	late adolescence	renal hematuria
	PTR2	male	7	early adolescence	anesthesia accident
	PTR3	female	40	old age	accident (drown)
Baboon	PHA1	male	30	adult	veterinary dispatched
	PHA2	male	9	subadult	veterinary dispatched
	PHA3	male	9	subadult	veterinary dispatched
Rhesus macaque	MMU1	female	24	old age	sepsis
Marmoset	CJA1	male	2	adult	veterinary dispatched
	CJA2	male	2	adult	veterinary dispatched
	CJA3	male	2	adult	veterinary dispatched
	CJA4	male	2	adult	veterinary dispatched
	CJA5	male	2	adult	veterinary dispatched

## Materials and Methods

### Tissue Samples, DNA and RNA Preparation

Human brain samples (excess material from autopsies) were obtained from the Department of Legal Medicine, University Medical Center Mainz, Germany in accordance with the principles of the Declaration of Helsinki. Primate brain samples were obtained from the Biomedical Primate Research Centre, Rijswijk, Netherlands and the German Primate Center, Göttingen with the support and through the European Primate Network EUPRIM-Net. Use of the human and anthropoid primate brain samples was approved by the Ethics Committee of the Medical Association of Rhineland-Palatinate (decisions 837.103.04 (4261) and 837.073.07 (5608)).

Samples were prepared between 1–2 days post-mortem from 14 humans (*Homo sapiens*, HSA), 3 common chimpanzees (*Pan troglodytes*, PTR), 3 hamadryas baboons (*Papio hamadryas*, PHA), 1 rhesus macaque (*Macaca mulatta*, MMU), and 5 white-tufted ear marmosets (*Callithrix jacchus*, CJA) (table 1). Age classes of humans [Knussmann, 1988], chimpanzees [Goodall, 1983], baboons [Sigg et al., 1982], rhesus macaque [Yu et al., 2011], and marmosets [Araujo et al., 2000] were determined according to established standards. Because of a similar brain architecture in humans and great apes [Semendeferi et al., 2001], area A10 was

excised from the frontal pole of human and chimpanzee brains. The baboon and marmoset samples were taken from the corresponding topological region. Frontal cortex tissue was immediately frozen and stored at  $-80^{\circ}\text{C}$  after dissection.

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The absorbance ratio at 260 nm and 280 nm was determined with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, Del., USA). All measured values were around 1.8, consistent with pure DNA preparations. Total RNA was isolated using TRIzol reagent (Invitrogen, Darmstadt, Germany). With the exception of 1 baboon sample, the absorbance ratios at 260 nm and 280 nm were between 1.92 and 2.04, indicative of pure high-quality RNA.

### Methylation Analyses

Human and primate sequences were retrieved from the Ensembl database and the NCBI BLAST archive. Evolutionarily conserved primers for Sanger sequencing of the target regions in the analyzed anthropoid primate species (table 2) were designed with the Primer3 program, version 0.4.0 (<http://frodo.wi.mit.edu/primer3/>). Following Exo/SAP digestion, the PCR products were sequenced on a Beckman Coulter CEQ 8000 Genetic Analysis System, using the CEQ DTCS Quick Start Kit (Beckman Coulter,

**Table 2.** Primers used for Sanger sequencing

Gene	Primer	Sequence (5'–3')	T <sub>m</sub> , °C	Amplicon length, bp	Localization on human chromosome 7, bp <sup>a</sup>
<i>MEST</i>	forward	GGGAGTAGCGGGGTCTTGG	63.1	718	130,131,701–130,132,418
	reverse	ATCCCGGGGTCTAGAAATCC	59.4		
<i>COPG2</i>	forward	TTCTGAAAAGCCACATTCACC	55.9	381	130,353,671–130,354,030
	reverse	ACTCCTCGTCCTTCTTGTCG	59.4		
<i>TSGA14</i>	forward	CCCATTTCTGCTGAACAACC	59.4	927	130,080,955–130,080,738
	reverse	ATCCCCACAATCAGAGATGC	59.8		

<sup>a</sup> According to Ensembl release 56 (September 2009).

**Table 3.** Primers used for bisulfite pyrosequencing

Gene	Primer	Sequence (5'–3')	T <sub>m</sub> °C	Amplicon length, bp	Number of CpGs
<i>MEST</i>	forward	YGATGGGYGGGTTAGGGG	60.5	296	6 (HSA, PTR, PHA, MMU) or 7 (CJA)
	reverse <sup>a</sup>	CATAACRCRRTTATCCCATACC	56.5		
	sequencing	GGTGGGTTTTAAAAG	42.4		
<i>COPG2</i>	forward <sup>a</sup>	TTTTTGGGAAGAKATGTTTATTAGAA	52.3	306	8 (HSA, PTR, PHA, MMU, CJA)
	reverse	RCRACAACAAAAATATAATTTTATT	49.9		
	sequencing 1	CACWACRAAACCTCC	50.5		
	sequencing 2	CTAAAAAWTCTATATTCACC	47.1		
<i>TSGA14</i>	forward	AGTAAAYGGGRTTTTTATTGGT	52.8	187	8 (HSA, PTR), 7 (PHA, MMU) or 6 (CJA)
	reverse <sup>a</sup>	ACCTCAAAATTCCTAATATACCT	55.3		
	sequencing 1	AYGGGTAAATYGGAGAAG	57.2		
	sequencing 2	GGGGTABGAAGTTAGG	50.0		

<sup>a</sup> Biotinylated.

HSA = *Homo sapiens*; PTR = *Pan troglodytes*; PHA = *Papio hamadryas*; MMU = *Macaca mulatta*; CJA = *Callithrix jacchus*.

Krefeld, Germany). Sequences were analyzed with the alignment tool in BioEdit (version 7.0.5.3) and the BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) program.

For methylation analyses, genomic DNA was bisulfite converted with the EpiTect Bisulfite Kit (Qiagen). Bisulfite PCR and pyrosequencing primers (tables 3, 4) were designed using the Pyrosequencing Assay Design Software (Qiagen). Gene-specific PCRs were carried out in 25 µl reactions with an initial denaturation step at 94°C for 3 min, 35 cycles of 94°C for 30 s, primer-specific annealing temperature for 30 s, 72°C for 60 s, and a final extension step at 72°C for 10 min. The reaction mixture consisted of 12.5 µl ReadyMix Taq PCR Reaction Mix with MgCl<sub>2</sub> (Sigma-Aldrich, Hamburg, Germany), 10 µl PCR grade water, 1 µl 10 pM forward primer and reverse primer each, and 1 µl DNA (100 ng/µl). Bisulfite pyrosequencing was performed on a PyroMark Q96 MD Pyrosequencing System (Qiagen) with the PyroMark Gold Q96 CDT Reagent Kit (Qiagen). The Pyro Q-CpG software (Qiagen) was used for data analysis. For quantitative methylation

data, we used box plots as graphics (IBM SPSS statistics) and the non-parametric Kruskal-Wallis test.

#### Expression Analyses

For microarray-based expression analyses, RNA was amplified with the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, Tex., USA). T7 promoter-containing cDNA was produced by reverse transcription of 400 ng total RNA each. This cDNA was subsequently transcribed into cRNA in the presence of biotinylated nucleotides. The labeled cDNA samples of different individuals from the same species (3 humans, 1 chimpanzee, 3 baboons, and 5 marmosets) were pooled. The 4 cDNA pools were hybridized under the same stringency conditions to 4 (of the 6) arrays on a Sentrix Human-6 Expression BeadChip (Illumina, San Diego, Calif., USA) that contain probes for >48,000 transcripts. Following washing, the arrays were stained with Cy3-streptavidin and scanned with an Illumina BeadStation 500. The Illumina BeadStudio software was used for data analysis. The 'rank invariant nor-

**Table 4.** Sequence analyzed by bisulfite pyrosequencing

Gene	Species	Sequence analyzed <sup>a</sup>
<i>MEST</i>	HSA, PTR, PHA, MMU CJA	TYGGTGTTTATTYGTTTYGYGTTGTGYGGTAATT TYGGYGTTTATTYGTTTYGYGGTYGYGGTAATT
<i>COPG2</i> (1)	HSA, PTR, MMU PHA CJA	CRTTACRAAAAACRCTCTCCCCRAACRCRATTTC CRTTACRAAAAACRCTCTCCCCRAACRCRATTTC CRTTACRAAAAACRCCCTCCCCRAACRCRATTTC
<i>COPG2</i> (2)	HSA, PTR, PHA, MMU, CJA	CRATAACCRTAACCT
<i>TSGA14</i> (1)	HSA, PTR PHA, MMU CJA	YGGTYGGGGAAGA YGGGTTAATYGGAGAAGA TGGTYGGGGAAGA YGGGTTAATYGGAGAAGG TGATYGGGGAAGATGGGTTAATYGGAGAAGA
<i>TSGA14</i> (2)	HSA, PTR, PHA, MMU, CJA	GTTAGAGGTAATTYGTGAGGTTAGAATTTYGAAYGTGGTYGGTTGGAG

<sup>a</sup> Analyzed CpG sites are underlined.

**Table 5.** Primers used for RT qPCR

Gene	Primer	Sequence (5'–3')	Tm, °C	Amplicon length, bp
<i>COPG2</i>	forward	CAATCCAAGAAGATGTTTGC	55.8	170
	reverse	CCATTTCTTTGATGGTAAGG	54.8	
<i>TSGA14</i>	forward	ACAAACCTTATCCTGACTGC	54.4	205
	reverse	CTGGCCAGCCTTTCAT	55.4	
<i>EIF2B2</i>	forward	TCAAGATTATCCGGGAGGAG	59.1	119
	reverse	ATGGAAGCTGAAATCCTCGT	58.7	
<i>YTHDC1</i>	forward	ATTATCATCTTCCGTTTCGTG	55.2	122
	reverse	CTTTGGCAAGAGACACATTC	55.9	

malization' algorithm was used for normalization of the data. A positive diffScore represents upregulation, while a negative diffScore represents downregulation. The p value for an observed expression difference between 2 analyzed groups of samples is <0.05 for genes with diffScores of lower than –13 and higher than +13 and <0.001 for diffScores of lower than –33 and higher than +33.

Quantitative real-time RT PCR was used to validate the differential mRNA expression of *COPG2* and *TSGA14* in human and anthropoid primate brains. One microgram of RNA sample was reversely transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Exon-spanning primers (table 5), showing 100% sequence conservation in all analyzed species, were designed with the Primer3 program. *EIF2B2* and *YTHDC1* were selected as endogenous control genes, because they showed stable mRNA levels in all analyzed brains in our array experiments and/or were previously identified as appropriate RT PCR control genes for evolutionary studies in primates [Fedrigo et al., 2010]. Samples of 2 human, 2 chimpanzee, 2 baboon, and 2 marmoset cortices were analyzed in triplicates using a Rotor-

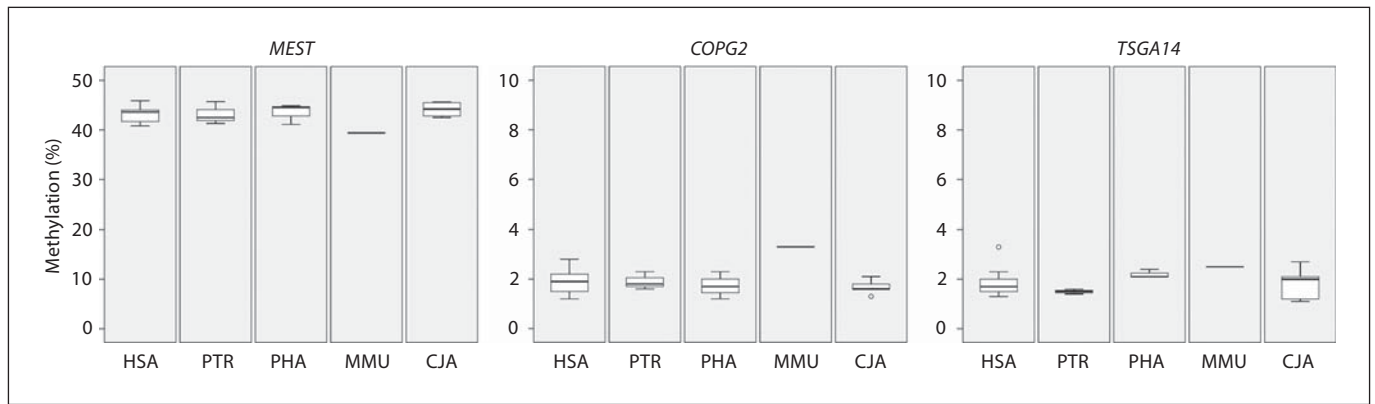
Gene Q system (Qiagen). The reaction mixture consisted of 10 µl SsoFast EvaGreen Supermix (BioRad, Munich, Germany), 7 µl PCR grade water, 1 µl each of forward and reverse primer, and 1 µl cDNA template. PCR was performed with 1 cycle of 98°C for 2 min and 40 cycles of 98°C for 5 s and 55°C for 20 s. Melting analysis was performed from 65°C to 95°C with 1°C increments for 5 s each. Data were analyzed with REST software, version 2.0.13 [Pfaffl et al., 2002].

## Results

### *Evolutionary Conservation of MEST, COPG2, and TSGA14 Methylation Patterns*

To test the hypothesis that adaptive changes in gene regulation during human brain evolution are a hallmark of genes underlying neurodevelopmental disorders, we





**Fig. 1.** Distribution of *MEST*, *COPG2*, and *TSGA14* promoter methylation values in 13 human, 3 chimpanzee, 3 baboon, 1 rhesus macaque, and 5 marmoset cortices. Medians are represented by horizontal lines. The bottom of the boxes indicates the 25th percentiles, the top the 75th percentiles. The T bars extend from the boxes to at most 1.5 times the height of the box. Outliers are shown as open circles.

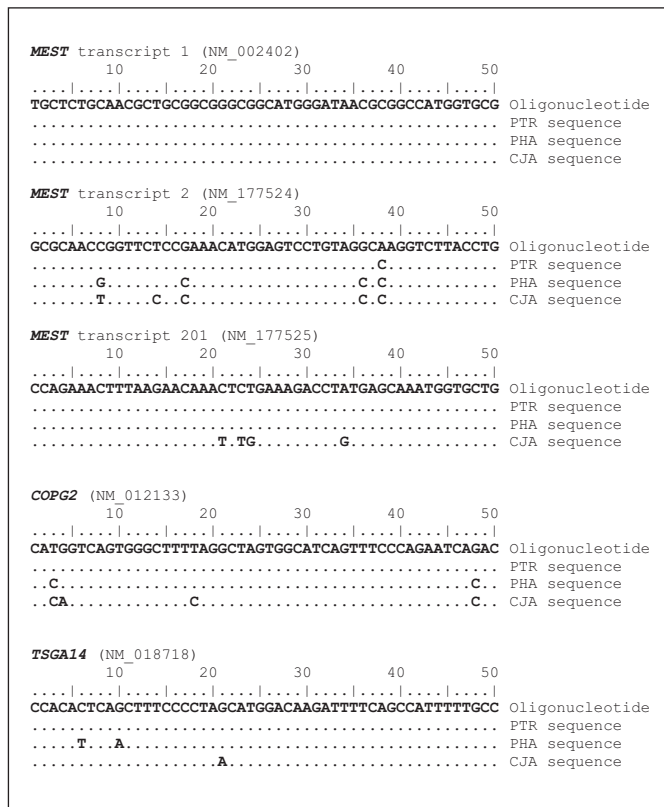
compared the methylation patterns of cis-regulatory regions in the 7q autism susceptibility locus in human and anthropoid primate cortices. We determined the promoter methylation levels of *MEST*, *COPG2*, and *TSGA14* in 13 human, 3 chimpanzee, 3 baboon, 1 rhesus macaque, and 5 marmoset cortices (table 1). To this end, we designed bisulfite pyrosequencing assays for orthologous regions in the *MEST*, *COPG2*, and *TSGA14* promoters of the 5 analyzed species. To exclude artifacts due to DNA sequence divergence, the target regions of the 3 studied genes were resequenced (by classic Sanger sequencing) in all analyzed brain samples and did not show any changes affecting our pyrosequencing assays (data not shown). Because methylated CpG sites are prone to transitional mutations (by deamination), the number of CpGs in the analyzed amplicons slightly varied between species due to evolutionary base changes. However, 6–8 CpGs were analyzed for each gene and species (tables 3, 4). Because the density of methylated CpGs rather than individual sites in a cis-regulatory region turn a gene on or off [Sontag et al., 2006; Weber et al., 2007], the average methylation of all CpGs in a given amplicon was taken as a representative epigenetic marker.

The box plots in figure 1 show the distribution of methylation values in all analyzed brain samples. The *MEST* promoter was hemimethylated as expected for the differentially methylated region of an imprinted gene. Mean methylation ( $\pm$  standard deviation) was  $43.2 \pm 1.6\%$  in human,  $43.2 \pm 2.3\%$  in chimpanzee,  $43.5 \pm 2.1\%$  in baboon,  $39.4\%$  in rhesus macaque, and  $44.1 \pm 1.5\%$  in marmoset cortices. There was no significant methylation

difference ( $p = 0.41$ ; Kruskal-Wallis test) between species. The *COPG2* and *TSGA14* promoters were hypomethylated, consistent with expression of these genes in the brain. *COPG2* methylation was  $1.9 \pm 0.5\%$  in humans,  $1.9 \pm 0.4\%$  in chimpanzees,  $1.7 \pm 0.6\%$  in baboons,  $3.3\%$  in rhesus macaque, and  $1.7 \pm 0.3\%$  in marmosets. *TSGA14* methylation was  $1.9 \pm 0.5$ ,  $1.5 \pm 0.1$ ,  $2.2 \pm 0.2$ ,  $2.5$ , and  $1.8 \pm 0.7\%$ , respectively. Again, there were no between-species differences ( $p = 0.48$  for *COPG2* and  $p = 0.19$  for *TSGA14*).

#### *Expression of MEST, COPG2, and TSGA14 in Human and Anthropoid Primate Cortices*

To assess gene expression of the 7q autism susceptibility locus, cDNA samples of 3 human, 1 chimpanzee, 3 baboon, and 5 marmoset cortices were hybridized on a human expression array. Because sequence mismatches between anthropoid primate samples and the human oligonucleotides may reduce the signal intensity, the interpretation of reduced expression signals (negative diffScores) in primates is difficult. In contrast, signals that appear to be upregulated (positive diffScores) in primates are unlikely to represent hybridization artifacts. The Illumina Expression BeadChip contains 50-bp oligonucleotides for 3 different isoforms of *MEST* (fig. 2). Transcript 2 (GenBank accession No. NM\_177524) was excluded from analysis because of too many sequence mismatches (1 in PTR, 4 in PHA, and 5 in CJA). The target sequence of transcript 1 (NM\_002402), which is located downstream and close to the analyzed promoter region, is perfectly conserved in all analyzed anthropoid primate



**Fig. 2.** Alignment of the human *MEST*, *COPG2*, and *TSGA14* oligonucleotide probes and the orthologous chimpanzee (PTR), baboon (PHA), and marmoset (CJA) sequences. For the anthropoid primate sequences, only the bases that differ from the human reference sequence are shown; base positions identical to the reference are indicated by dots.

species; the sequence of transcript 201 (NM\_177525) is conserved in chimpanzee and baboon but shows 4 mismatches in marmoset. The diffScores for *MEST* transcripts 1 and 201 varied between  $-3.5$  and  $+2.7$  in the 3 analyzed anthropoid primate species which strongly argues against differential expression. Compared to humans, the analyzed *COPG2* transcript (NM\_012133) was possibly upregulated in the chimpanzee ( $p < 0.05$ ; diffScore 16) and significantly upregulated in baboon ( $p = 3.38 \times 10^{-12}$ ; diffScore 114) and marmoset ( $p = 3.68 \times 10^{-38}$ ; diffScore 374). The analyzed *TSGA14* transcript (NM\_018718) was possibly downregulated in the chimpanzee ( $p < 0.05$ ; diffScore  $-14$ ) and baboon ( $p < 0.001$ ; diffScore  $-37$ ). However, as outlined above, this downregulation should be interpreted with caution because of the relatively high  $p$  value (not corrected for multiple testing) in the chimpanzee and 2 sequence mismatches in the

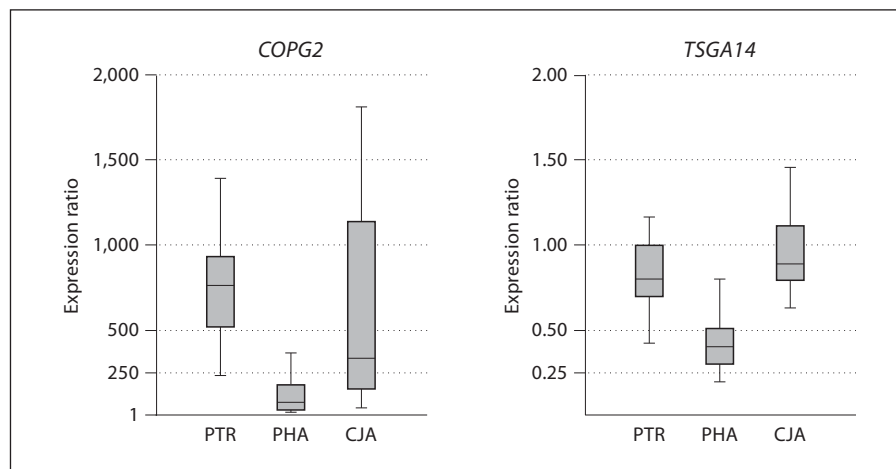
baboon. The marmoset showed significant upregulation ( $p = 2.89 \times 10^{-15}$ ; diffScore 145).

To validate differential expression of *COPG2* and *TSGA14* in catarrhine and New World monkeys, we designed quantitative real-time RT PCR assays using primer sequences (table 5) which have been conserved in all analyzed species. Consistent with the array results, RT qPCR of 2 human, 2 chimpanzee, 2 baboon, and 2 marmoset cortices revealed a significant upregulation of *COPG2* mRNA in all analyzed anthropoid primate species, compared to humans (fig. 3). The PTR/HSA ratio (by REST analysis) was 661 ( $p = 0.003$ ; SE range 421–1,007), the PHA/HSA ratio 67 ( $p = 0.001$ ; SE range 24–243), and the CJA/HSA ratio 370 ( $p = 0.001$ ; SE range 115–1,434). For *TSGA14*, RT qPCR revealed comparable expression levels in the chimpanzee (PTR/HSA ratio 0.81;  $p = 0.09$ ; SE range 0.67–1.13) and the marmoset (CJA/HSA ratio 0.92;  $p = 0.45$ ; SE range 0.73–1.18), whereas in the baboon it appeared to be slightly downregulated (PHA/HSA expression ratio 0.39;  $p = 0.002$ ; SE range 0.25–0.59) (fig. 3). The conflicting results between RT qPCR and expression arrays (which showed an upregulation of *TSGA14* in marmoset cortices) can be explained by the fact that arrays recognize only 1 specific isoform, whereas RT qPCR quantifies multiple isoforms of the same gene. In addition to the *COPG2* isoform NM\_012133 targeted by the Illumina oligonucleotide, our exon-spanning RT-PCR primers also amplify transcripts ENST00000445977 and ENST00000330992. For *TSGA14*, the Illumina oligonucleotide hybridizes only with transcript NM\_018718, whereas RT qPCR quantifies 6 different transcripts (NM\_018718, ENST00000541543, ENST00000484549, ENST00000343969, ENST00000480206, and ENST00000492389). Collectively, our data suggest that *MEST* is not differentially expressed in human and anthropoid primate cortices, whereas *COPG2* is downregulated in human cortex compared to all analyzed anthropoid primate species.

## Discussion

Numerous comparative genome-wide expression studies have identified gene expression differences between human and non-human primates which may have evolved under natural selection and contributed to phenotypic differences [Enard et al., 2002; Khaitovich et al., 2005, 2006; Nowick et al., 2009]. Methylation of CpG dinucleotides in the cis-regulatory regions of promoters is generally thought to act as an epigenetic signal that regu-

**Fig. 3.** Box plots showing ratio and range of *COPG2* and *TSGA14* expression in anthropoid primate versus human cortices. Bottom and top of the boxes indicate the 25th and the 75th percentiles, respectively. Horizontal lines represent the median determined by REST analysis of real-time RT PCR data. T bars represent the minimum and maximum of 10,000 iterations performed. An expression ratio of 1 is consistent with equal expression levels in the 2 analyzed species; values <1 and >1 indicate down- and upregulation, respectively, in the analyzed anthropoid primate compared to the human cortex.



lates gene expression. Genome-wide methylation profiling revealed that tissue-specific methylation patterns are highly conserved between human and chimpanzee [Martin et al., 2011; Molaro et al., 2011; Pai et al., 2011] and even between human and mouse [Eckhardt et al., 2006]. Only about 150 promoter regions (representing <1% of all studied genes) were found to be differentially methylated in human and chimpanzee sperm [Molaro et al., 2011]. In different somatic tissues only 10–20% of tissue-specifically methylated regions showed methylation differences between humans and chimpanzees [Pai et al., 2011]. It is estimated that differential methylation at promoter regions accounts for only a minor part of gene expression differences between primate species. There are relatively few studies comparing methylation patterns of specific genes in primate cortices. An array-based comparison of 145 CpG sites from 36 genes identified 18 CpGs (12 genes) that have differences in methylation between humans and chimpanzees, mainly in the brain [Enard et al., 2004]. Comparative bisulfite sequencing revealed differential methylation of an ALU repeat in the *CCRK* promoter in human, chimpanzee, and Old World monkey cortices, associated with gene expression differences [Farcas et al., 2009]. In this study we found that the methylation patterns of 1 imprinted gene, *MEST*, and 2 closely adjacent/overlapping non-imprinted genes, *COPG2* and *TSGA14*, in the 7q autism susceptibility locus are highly conserved in human, chimpanzee, Old World monkey (baboon and rhesus macaque), and New World monkey (marmoset) which diverged more than 40 million years ago [Steiper and Young, 2006].

Our observation that the *MEST* promoter is hemimethylated in human and anthropoid primate cortices

confirms previous studies that *MEST* is imprinted in humans [Nishita et al., 1996] and other mammals (<http://igc.otago.ac.nz>; <http://www.geneimprint.org>). Earlier genome-wide expression analyses [Khaitovich et al., 2005; Nowick et al., 2009] already suggested that *MEST* is not differentially expressed in the human and chimpanzee brain. We show that the *MEST* expression level remained unchanged in the anthropoid primate lineage leading to humans. As expected for a paternally expressed gene, *MEST* plays a role in resource acquisition of the fetus and promotes growth [Lefebvre et al., 1998]. In adult mice, upregulation of *MEST* expression is associated with enlargement of adipocytes and expansion of white adipose tissue [Takahashi et al., 2005] which serves as an energy reservoir. Consistent with the view that imprinted genes are also important for the allocation of parental resources after birth and development of a ‘social brain’ [Ubeda and Gardner, 2010, 2011], the paternally expressed *MEST* gene was shown to underlie maternal care in mice [Lefebvre et al., 1998]. However, the conserved methylation and expression patterns of *MEST* in human and anthropoid primate cortices do, at least, not support the assumption that changes in *MEST* regulation contributed to the evolution of human cognitive and/or advanced communication abilities.

The *COPG2* gene was hypomethylated (1.7–3.3%) in all analyzed cortex samples. Evidently, *COPG2* is not imprinted in human and anthropoid primate brains. *COPG2* is biallelically expressed in fetal and adult human brain. However, whether or not it is maternally imprinted (paternally expressed) in other fetal human tissues remains controversial [Blagitko et al., 1999; Yamasaki et al., 2000]. In the mouse, *Copg2* appears to be paternally im-



printed (maternally expressed) in embryos and adult brain [Lee et al., 2000]. It is tempting to speculate that changes in *COPG2* regulation are associated with phenotypic evolution at the organismal level. Despite comparable promoter hypomethylation, *COPG2* mRNA expression was downregulated in the human cortex compared to all analyzed anthropoid primate species. Although we cannot exclude the possibility that we studied CpG sites outside the relevant cis-regulatory region, *COPG2* may belong to a growing group of genes for which 5' promoter methylation does not play a major role in the regulation of expression [Weber et al., 2007; Maunakea et al., 2010]. Other mechanisms, i.e. gene body methylation, may be more important for the species-specific efficiency of transcription. Genes with stable expression levels among non-human primates and up- or downregulation in the human lineage are good candidates for disease association [Gilad et al., 2006]. In this light, *COPG2* is an interesting candidate gene for ASD and human-specific communication abilities. The imprinted brain hypothesis of autism proposes that imbalances between paternally and maternally expressed genes skewed to the paternal side impair social cognition which is underdeveloped in ASD [Badcock and Crespi, 2006]. Although *COPG2* is not imprinted in human and anthropoid primate brains, it is interesting to note that the 'social human brain' shows a lower expression than anthropoid primate brains. The human frontal cortex displays a higher ratio of glia to neurons than other primates [Sherwood et al., 2006]. However, these minor differences in cell composition cannot explain the observed dramatic differences in *COPG2* expression between human and anthropoid primate cortices.

Similar to *COPG2*, the *TSGA14* promoter was hypomethylated (1.5–2.2%) in human and anthropoid primate cortices as expected of a transcriptionally active non-imprinted gene. The expression analyses yielded conflicting

results. The significant upregulation in the marmoset brain seen by microarrays could not be confirmed by RT qPCR. However, considering that hybridization-based interspecific comparisons are more susceptible to artifacts (i.e. due to sequence divergence), we mainly relied on the results of qPCR which quantifies a much larger number of transcripts. Using RT qPCR, *TSGA14* either showed a slight upregulation of expression in the human brain (compared to baboon) or no differential regulation (compared to chimpanzee and marmoset). An earlier study [Khaitovich et al., 2005] also reported comparable expression levels in human and chimpanzee brains. *TSGA14* has a short testis-specific isoform and a long ubiquitously expressed isoform with particularly high expression in the brain [Yamada et al., 2002]. Its DNA sequence was highly conserved during primate evolution with a dN/dS ratio of 0.1 for the chimpanzee and 0.3 for the marmoset (Ensembl version 65, Dec. 2011). The long isoform encodes a protein which binds to the centrosome, a microtubule-organizing center which plays an important role in neuronal development [Higginbotham and Gleeson, 2007]. Recently, rare potentially pathogenic *TSGA14* sequence variants were found to be enriched in patients with familial ASD [Korvatska et al., 2011], supporting a link between *TSGA14* and human communication abilities.

Collectively, our results suggest that DNA methylation at 3 promoter regions in the autism susceptibility locus on human chromosome 7q32 has been highly conserved during primate evolution. Nevertheless, one gene, *COPG2*, shows human-specific downregulation in the brain. Evidently, DNA methylation is not the only mechanism underlying intra- and interspecific differences in gene regulation. We propose that adaptation of *COPG2* expression may be associated with the evolution of a 'social brain' and, consequently, its misregulation may play a role in disorders of the 'social brain' such as ASD.

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